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ORIGINAL ARTICLE

Immunomodulatory Effect of *Kaempferia parviflora* against Cyclophosphamide-Induced Immunosuppression in Swiss Albino Mice

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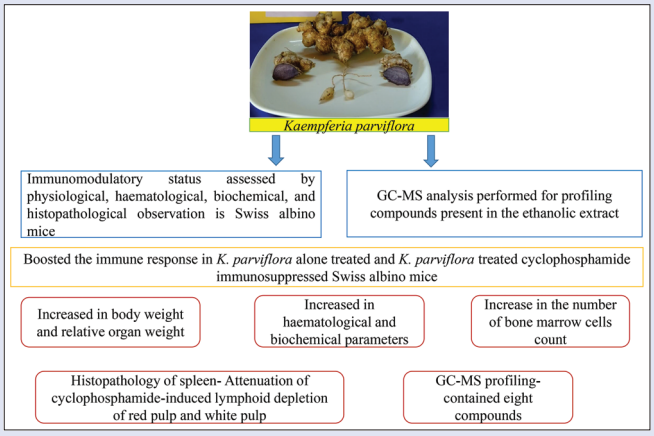
ABSTRACT

Background: *Kaempferia parviflora* Wall. Ex. Baker (Black ginger) is an important medicinal plant used as a health-promoting tonic i.e., both a stimulant and a vitalizing agent in Thailand. Research findings are available on its aphrodisiac and anti-inflammatory activities. So far, there is no research work conducted on immunomodulatory effect of *K. parviflora*. **Objectives:** The present study was carried out to evaluate the immunomodulatory effect of ethanolic extract of rhizomes of *K. parviflora* in cyclophosphamide-induced immunosuppression in Swiss albino mice. **Materials and Methods:** Immunomodulatory status was assessed by physiological, hematological, biochemical, and histopathological observations. The weight of organs such as liver and spleen was recorded at the time of sacrifice. Gas chromatography mass spectrophotometry (GC-MS) analysis was performed for profiling compounds present in the extract. **Results:** Significant increase in body weight was observed on 12th day in *K. parviflora*-treated immunosuppressed mice. In hematological parameters, there was significantly higher lymphocyte count for *K. parviflora*-treated immunosuppressed mice. In hemagglutination test, conducted for the evaluation of humoral immune response, both *K. parviflora* alone and *K. parviflora*-treated immunosuppressed mice showed significant increase in titer value compared with cyclophosphamide control. Bone marrow cellularity test performed for evaluation of cellular immune response showed cyclophosphamide control group with significant lower bone marrow cellularity on 12th and 19th day while *K. parviflora* alone-treated and *K. parviflora*-treated immunosuppressed mice showed a significant increase in the bone marrow cellularity. The result of histopathology of spleen revealed to prevent the depletion of red pulp and white pulp on 12th day, and this prevention was marked on 19th day. GC-MS profiling showed that the extract contained eight compounds. Majority of the compounds belong to flavonoids class which might have helped in immunomodulation. **Conclusion:** The results of the present study revealed that the test extract possessed promising immunomodulatory activity.

Key words: Biochemical, cellular response, gas chromatography mass spectrophotometry, hematological, humoral response, *Kaempferia parviflora*

SUMMARY

- The present study revealed the administration of ethanolic extract of *Kaempferia parviflora* boosted the immune response in *in vivo* experiment in Swiss albino mice
- In terms of increase in body weight and increase in relative organ weight
- The plant extract increased body weight, relative organ weight and increased hematological parameters, namely total leukocyte and differential leukocyte counts, increase in titer value in hemagglutination test
- The plant extract increased the number of bone marrow cells count, serum protein and globulin
- Attenuation of cyclophosphamide-induced depletion of red pulp and white pulp in histopathology of spleen and the significant increase in footpad thickness of *K. parviflora* alone-treated group and *K. parviflora*-treated cyclophosphamide immunosuppressed group when compared with cyclophosphamide control was also observed.



Abbreviations used: GC-MS: Gas chromatography mass spectrophotometry; SRBC: Sheep Red Blood Cells; PBS: Phosphate buffer saline; HA: Hemagglutination assay; DTH: Delayed-type hypersensitivity; HSC - hematopoietic stem cells.

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INTRODUCTION

The medicinal *Kaempferia* species are the rhizomatous herbs belonging to the family *Zingiberaceae*. *Kaempferia parviflora* Wall. Ex Baker, popularly known as black ginger or Thai ginger, is indigenous to the northeastern part of Thailand. The herb is locally known as *Krachaidum* in Thailand and as *Khongban Takhellei* in Manipur.[1] The plant is about 30–40 cm tall. The number of leaves varies from 1 to several; blades are ovate or oblong

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shape and slightly unequal sided, upper surface are yellow green and the lower surface are green in colour. The rhizome is subglobose with several succulent roots in a fascicle. It has brownish skin with purple color interior flesh. Its inflorescence is enclosed by two innermost leaf sheaths.[2] Rhizomes of *K. parviflora* have been used as traditional medicine for rectifying male impotence, body pains, and gastrointestinal disorders among local people in the Northeast of Thailand.[3] In India, it has been reported to occur in the tropical evergreen forest of Imphal East district of Manipur.[1]

In Thailand, *K. parviflora* is well known as an energy enhancer with exceptional tonic effect. Fresh or dried rhizomes and dried powder in tea bag and wine are various products used by inhabitants of Thailand. Alcoholic infusion of *K. parviflora* rhizome has been used as a tonic for body pains and gastrointestinal disorders.[4] Rhizome is reported to have antimicrobial, aphrodisiac, antigastric ulcer, antidepressant, anticholinesterase activity, antiobesity, vasodilation, and antioxidant effect. Traditional medicines using *K. parviflora* are permitted by Thai Food and Drug Administration which include capsules, pills, tablets, powders, and essence tincture.[5] Various *in vivo* experiments in the test animals using *K. parviflora* extract showed reduction in obesity, diabetes Type II, cardiovascular disease, and inflammatory activity.[6,7]

There are reports available on the effect of *K. parviflora* in strengthening the body in general and as sexual stimulant by the consumption of rhizome extracted with alcohol.[8] Increased whole-body energy expenditure in healthy men by the ethanolic extract may be useful as an antiobesity regimen.[9] Wattanathorn *et al*. also reported about the enhanced male sexual behaviors in aging rats by the administration of crude extract of this plant.[10] Another study in male mice showed that *K. parviflora* improved physical fitness performance and muscular endurance.[11] It also acts as modulator of multidrug resistance in cancer cells.[12] Adaptogenic activities of *K. parviflora* has been reported in mice.[13] All these studies indicate the general health-promoting effect of *K. parviflora*.

Many *in vitro* studies have shown anticancer activities of *K. parviflora* extract, i.e., *K. parviflora* extract was cytotoxic to SKOV3 cells,[14] 5a-reductase (5aR),[15] and human cholangiocarcinoma cell lines (HuCCA-1 and RMCCA-1).[16] Recent findings showed that *K. parviflora* rhizomes extract contained numerous flavonoids,[17] which was previously reported to possess antioxidant activity.[18,19] Plants which possessed anticancer and antioxidant properties are reported to have immunomodulatory property.[20,21]

The major components of *K. parviflora* volatile oil are α-copaene, dauca-5, 8-diene, camphene, β-pinene, borneol, and linalool while the hexane extract showed germacrene D, β-elemene, α-copaene, and E-caryophyllene as major constituents.[21,22]

Even though cyclophosphamide is a widely used as an alkylating drug for the treatment of various types of cancers such as lymphoma, myeloma, and chronic lymphocytic leukemia, it is having effective immunosuppressive action which cross links the DNA of actively dividing cells thereby inhibiting the both cellular and humoral response immunity.[23] Cyclophosphamide can be used as immunosuppressive agent to study the immunomodulatory effects of plant extracts by various researchers.[24,25]

Precise information on the immunomodulatory properties are lacking for *K. parviflora*. Hence the present study was carried out to evaluate the immunomodulatory effect of ethanolic extract of the rhizome of *K. parviflora* in cyclophosphamide-induced immunosuppression model in Swiss albino mice.

MATERIALS AND METHODS

Plant material

The rhizomes were collected from the field experiments conducted at field of the Department of Plantation Crops and Spices, Kerala Agricultural University, Vellanikkara. Identification was done by Dr. A. A. Mao (Scientist E), Botanical Survey of India, Eastern Regional Centre, Shillong. A herbarium was prepared, and voucher specimen with accession no. HERB/VPT/CVASMTY/3/2019 was deposited at Department of Veterinary Pharmacology and Toxicology, College of Veterinary and Animal Sciences, Mannuthy.

Preparation of extracts

The rhizomes were shade dried followed by course pulverization using mechanical pulverizer. Plant extract was obtained using 95% ethanol by soxhlet extraction. The extract was then dried using rotary evaporator.

Experimental design

Experiment was conducted at Department of Veterinary Pharmacology and Toxicology, College of Veterinary and Animal Sciences, Mannuthy. The experiment protocol was approved by Institutional Animal Ethics Committee of College of Veterinary and Animal Sciences, Mannuthy (Order no. IAEC/CVASMTY 4/17-18). Swiss albino mice, procured from Small Animal Breeding Station, Mannuthy were randomly divided in two sets of 48 each. First set of animals (Group A) were used for physiological, hematological, biochemical, and bone marrow cellularity tests with four subgroups (A_I, A_{II}, A_{III}, and A_{IV}) of 12 animals each. Second set of animals (Group B) were used for delayed hypersensitivity with four subgroups (B_I, B_{II}, B_{III}, and B_{IV}) of 12 animals each. Sheep Red Blood Cells (SRBC) antigen (1×10^8 cells/mL/100 Kg BW) were injected i. p. to mice of all the groups on 5th day except A_I and B_I. The experiment protocol is illustrated in Table 1.

Immunization

Blood was collected from the sheep maintained within the University Sheep and Goat farm, Mannuthy, in equal volume of Alsever's solution following sterile procedure. This was used for antigen preparation and stored at 4°C until used.

Measurement of physiological parameters

The weight of individual mouse was recorded before, during (on 12th day), and at the end (19th day) of the experiment. The weight of the organs such as spleen and liver were conjointly recorded at the time of sacrifice.

Table 1: Experiment protocol

Group	Treatments
A_I and B_I	Control group receiving vehicle for 19 days
A_{II} and B_{II}	Mice administered with cyclophosphamide @ 30 mg/kg BW on 9th, 10th, 11th, 16th, 17th, and 18th day
A_{III} and B_{III}	Mice administered with ethanolic extract of rhizome of *K. parviflora* @ 200mg/Kg orally for 19 days
A_{IV} and B_{IV}	Mice administered with ethanolic extract of rhizome of *K. parviflora* @ 200mg/Kg orally for 19 days and cyclophosphamide @ 30 mg/kg BW on 9th, 10th, 11th, 16th, 17th, and 18th day

BW: Body weight; *K. parviflora*: *Kaempferia parviflora*

Measurement of hematological parameters

Blood samples were collected from submaxillary vein of all the mice from group A_I to A_{IV} on zero, 12th, and 19th day of the experiment. To prevent variations, blood samples were placed in ethylene diamine tetraacetic acid tubes for analysis. [26] Hematology analyser, model-Mythic 18 Vet (Orphee, Switzerland) was used for the analysis of total and differential leukocyte count.

Measurement of biochemical parameters

Blood collected from submaxillary vein of mice from group A_I to A_{IV} was taken test tube without anticoagulant, centrifuged at 2500 rpm for 10 min. Serum was separated for the estimation of protein, albumin, and hemagglutination test. Biochemical analysis was done using Semi-Automated Biochemical Analyser, model-Master T (Hospitex, Italy).

Measurement of immunological parameters

Hemagglutination test

Hemagglutination test was performed for the evaluation of cell-mediated immune response. The blood was collected from mice in the groups A_I to A_{IV} on zero, 12th, and 19th day of the experiment. Two-fold dilutions of sera were prepared in 0.15 M phosphate buffer saline (PBS) (PH 7.2), and 50 µl of each dilution was transferred into 96 well microtiter plates. Twenty-five microliter quantity of fresh one per cent Sheep SRBC suspension in PBS was added into each well and mixed thoroughly. Thereafter, they were incubated at 37°C for 1 h. The reciprocal of highest dilution of the test serum giving 50% agglutination had been expressed as hemagglutination assay titer.[27]

Bone marrow cellularity

Bone marrow cellularity was done for the evaluation of humoral immune response. Femurs of both the hind legs of the mice were dissected, and the condyles of the femurs were removed using sharp scissors.[28] Then, the bone marrow was flushed with 5 mL of 10% fetal bovine serum. The number of cells was counted hemocytometrically using invitrogen countess automated cell counter on 12th day and 19th day after sacrificing the animals.

Histopathology of spleen

On 12th and 19th days, the spleen of the animal was removed for histopathology. The tissue samples were fixed at 10% formalin and embedded in paraffin and the sections were stained using hematoxylin and eosin.[29] It was observed for histopathological lesions.

Delayed type hypersensitivity

Six mice from each groups of B_I, B_{II}, B_{III}, and B_{IV} were primed with SRBC antigen i. p. on day 5 and was then challenged on day 12 with SRBC antigen s. c. on the right hind footpad. The left hind foot pad received 0.025 mL of saline alone. The footpad swelling was measured at three different dimensions using Vernier calipers after 24 h of challenge. The difference in footpad thickness was taken as a measure of delayed-type hypersensitivity. The test was repeated on 19th day in the remaining six mice of each group.[30]

Gas chromatography mass spectrophotometry analysis

The active phytochemical principles of *K. parviflora* was analyzed using Shimadzu gas chromatography mass spectrophotometry (GC-MS) (Model Number: QP2010S), Kerala Forest Research Institute, Peechi, Thrissur, Kerala. The oven temperature was maintained at 70°C for 2 min and then increased to 200°C in 5 min. The injector temperature was 260°C, and total analysis time was 50 min. One microliter aliquots of extracts were injected into the chromatographic capillary column of length 30 m, inner diameter 0.25 mm, and film thickness 0.25 µm after a clear baseline had been obtained. Major constituents were identified using mass spectrum NIST 11 and WILEY.

Statistical analysis

Analysis of covariance was done for hematological parameters and two factor analysis for organ weight and biochemical parameters. $P < 0.05$ was considered to be significant.

RESULTS

Body weight

The relative change in body weight from 0th to 12th day and 12th to 19th days are given in Table 2. There was significant increase in body weights on 12th day of experiment in group A_I, A_{III}, and A_{IV}. There were significant reduction in body in group A_{II} in both the first six set of animals and second six set of animals. The body weights of mice in *K. parviflora*- and cyclophosphamide-treated group (A_{IV}) showed a significant ($P < 0.05$) increase on 12th day of the experiment compared with cyclophosphamide control (A_{II}). The highest relative increase in body weight was recorded on A_{III} with mean value of 9.074 ± 0.66 g while the relative decrease in body weight was lowest in A_{II} with mean value of −6.276 ± 0.41 g.

Organ weight

The weight of internal organs such as spleen and liver were taken on 12th and 19th day of the experiment and expressed as relative organ weights [Table 3]. There was no significant difference in liver weight on 12th and 19th day. On 12th day, both *K. parviflora* alone treated (A_{III}) and *K. parviflora* treated immunosuppressed animals (A_{IV}) showed significant increase in spleen weight compared with normal control (A_I) and cyclophosphamide control (A_{II}). *K. parviflora* treated immune suppressed animals (A_{IV}) showed significantly higher spleen weight on 19th day.

Hematological parameters

Total leukocyte count recorded on zero, 12th, and 19th day of the experiment is presented in the Table 4. On 12th day, *K. parviflora*-treated immunosuppressed group showed significantly higher leukocyte count compared with cyclophosphamide- and normal-treated groups. On 19th day, significant increase in leukocyte count was observed in A_{III} and A_{IV}. The lymphocyte count showed significant difference on 12th and 19th day of the experiment [Table 5]. On 12th day, A_{IV} showed significant higher value compared with A_I and A_{II}. On 19th day, A_{III} and A_{IV} showed significant increase in lymphocyte count compared with A_{II}.

There was no significant difference in monocyte count on 12th day of the experiment. *K. parviflora*-treated immunosuppressed group showed significant increase in monocyte count on 19th day of the experiment as compared with cyclophosphamide control [Table 5]. There was no significant difference in neutrophil count on 12th day of the experiment. Neutrophil was found significantly highest in A_{II} on 19th day [Table 5].

Immunological parameters

Hemagglutination titer was taken on 0th, 12th, and 19th day [Table 6]. Both A_{III} and A_{IV} showed significant increase in titer value on 12th day with titer value of 938.667 and 938.667 respectively.

Table 2: Effect of ethanol extract of *Kaempferia parviflora* in relative change in body weight in cyclophosphamide immunosuppressed Swiss albino mice, g

	Days	A_I	A_{II}	A_{III}	A_{IV}
First six animals	12th day	4.519±0.61	−6.276±0.41	9.074±0.66[a]	3.279±0.23[a]
Second six animals	12th day	7.196±0.55	−4.530±0.54	5.452±0.36[b]	1.349±0.20[b]
	19th day	8.257±0.73	−2.658±0.29	2.109±0.28[c]	1.387±0.18[c]

Value are expressed as mean±SE. *n*=6 Swiss albino mice, per group, [a]*P*<0.05 when compared with cyclophosphamide control (12 days) for first six animals; [b]*P*<0.05 when compared with cyclophosphamide control (12 days) for second six animals; [c]*P*<0.05 when compared with cyclophosphamide control (12 days) for second six animals. SE: Standard error

Table 3: Effect of ethanol extract of *Kaempferia parviflora* on the liver and spleen weight in cyclophosphamide immunosuppressed Swiss albino mice, g/100

Organs	Days	A_I	A_{II}	A_{II}	A_{IV}
Liver	12th day	5.652±0.44	4.761±0.23	6.824±0.38	5.901±0.37
	19th day	5.087±0.31	4.579±0.10	5.469±0.23	6.401±0.52
Spleen	12th day	0.434±0.05	0.422±0.03	0.656[a]±0.03	0.585[a]±0.03
	19th day	0.458±0.04	0.453±0.01	0.490[b]±0.03	0.596[b]±0.03

Liver: Value are expressed as mean±SE. *n*=6 Swiss albino mice, per group, mean value do not differ significantly. Spleen: Value are express in mean±SE. *n*=6 Swiss albino mice, per group, [a]*P*<0.05 when compared with cyclophosphamide control (12 days); [b]*P*<0.05 when compared with cyclophosphamide control (19 days). SE: Standard error

Table 4: Effect of ethanol extract of *Kaempferia parviflora* on the total leukocyte count in cyclophosphamide immunosuppressed Swiss albino mice, 10^3/µl

Groups	12th	19th
A_I	5.597±0.48	5.388±0.35
A_{II}	4.463±0.48	3.442±0.22
A_{III}	4.622±0.18	10.293±0.51[b]
A_{IV}	7.968±0.36[a]	6.102±0.37[b]

Value are expressed as mean±SE. *n*=6 Swiss albino mice, per group, [a]*P*<0.05 when compared with cyclophosphamide control (12 days); [b]*P*<0.05 when compared with cyclophosphamide control (19 days). SE: Standard error

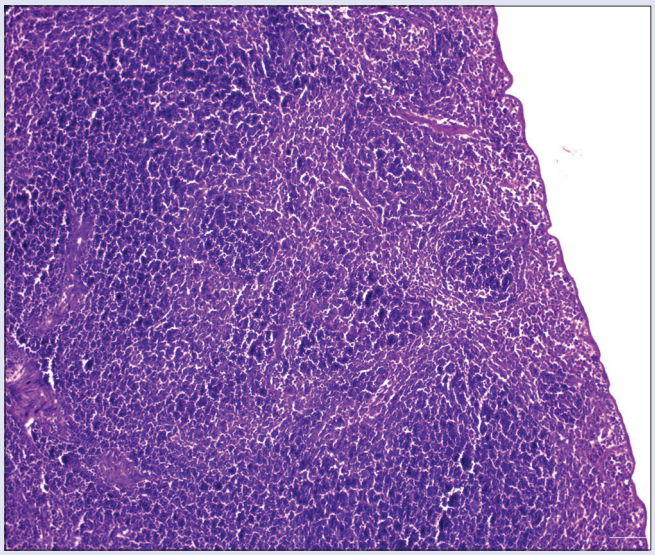

Figure 1: Histopathology of spleen of Swiss albino mice in control group receiving vehicle at 12th day. Light microscopic image of H and E-stained sections of spleen revealed red pulp and white pulp packed with lymphocytes (×100)

Cyclophosphamide control group (A_{II}) showed significant lower bone marrow cellularity on 12th and 19th day compared with normal control (A_I). Both A_{III} and A_{IV} showed significantly higher value compared with A_I and A_{II} on 12th and 19th day [Table 7].

Biochemical parameters

Total protein and globulin

Total protein was observed lowest in A_{II} on 12th and 19th day [Table 8]. There were significant increase in total protein in *K. parviflora* alone-treated (A_{III}) and *K. parviflora*-treated immune suppressed mice (A_{IV}) when compared with cyclophosphamide control (A_{II}). There was no significant difference in globulin content. On 12th day, globulin content was highest in A_{IV}.

Histopathology of spleen

Marked lymphoid depletion was observed in white pulp and marginal zone of spleen in cyclophosphamide-induced mice as compared with normal control animals [Figures 1-4]. Administration of *K. parviflora* induced hyperplasia in white pulp region [Figures 5 and 6]. However, administration of *K. parviflora* along with cyclophosphamide-induced mice revealed attenuated lymphocyte depletion on 12th day and marked attenuation of lymphocyte depletion on 19th day [Figures 7 and 8].

Delayed type hypersensitivity

The increased in foot pad thickness of all the experimental animals were observed on 12th and 19th day [Table 9]. Both B_{III} and B_{IV} showed significant increase in footpad thickness on 12th and 19th day.

Gas chromatography mass spectrophotometry analysis

Phytoconstituents obtained on GC-MS analysis of *K. parviflora* is listed in Table 10 and Figure 9. A total of eight compounds in ethanolic extract were identified. The majority of the compounds detected were belong to flavonoids and fatty acids. The major compound found were dimethylchrysin (74.43%) and techtochrysin (8.79%).

DISCUSSION

In the present study, SRBC antigen (1×10^8 cells/mL/100 Kg BW) were injected i. p. to mice of all the groups on 5th day except A_I and B_I. The dose was selected as per Tizard.[31] Two hundred milligram per kilogram of ethanolic extract was administered to mice for 19 days. The dose was selected as per the toxicity study (unpublished data). The toxicity studies of *K. parviflora* was conducted and found that the plant extract was not toxic at 2000 mg/Kg. Again, reports are available on the administration of dose of 200 mg/Kg body weight to Swiss albino mice in in vivo studies of the immunomodulatory effects of methanol leaf extract of *Gymnema sylvestre*,[32] aqueous and ethanolic extract of dried tuber of *Eulophia nuda*.[33] Acute and chronic toxicity studies of, acute and chronic toxicity study of *Kaempferia parviflora* powder was reported by Chivapat *et al.*[34] There is a thumb rule that a safe upper limit for selecting a dose is 10% of LD_{50}, when no experimental data existed.[35] Considering the cited reports and thumb rule, we have selected the dose of 200 mg/Kg.

Swiss albino mice, used in the study, are immunocompromised mice. Hence, to study the immunomodulatory effect of plant extracts, Swiss

Table 5: Effect of ethanol extract of *Kaempferia parviflora* on the lymphocyte, monocyte and neutrophil in cyclophosphamide immunosuppressed Swiss albino mice

Groups	Hematology parameters					
	Lymphocyte (%)		Monocyte (103/µl)		Neutrophil (%)	
	12th	19th	12th	19th	12th	19th
A_I	5.289±0.25	5.141±0.11	0.449±0.04	0.422±0.05	0.472±0.04	0.525±0.05
A_{II}	3.443±0.31	3.638±0.19	0.422±0.04	0.367±0.03	0.554±0.06	0.695±0.04[b]
A_{III}	4.135±0.11	8.073±0.56[b]	0.588±0.04	0.447±0.05	0.249±0.02	0.445±0.04
A_{IV}	6.683±0.29[a]	5.532±0.16[b]	0.425±0.03	0.664±0.03[b]	0.343±0.03	0.452±0.04

Lymphocyte: Value are expressed as mean±SE. n=6 Swiss albino mice, per group, [a]P<0.05 when compared with cyclophosphamide control (12 days); [b]P<0.05 when compared with cyclophosphamide control (19 days). Monocyte: Value are expressed as mean±SE. n=6 Swiss albino mice, per group; [b]P<0.05 when compared with Cyclophosphamide control (19 days). Neutrophil: Value are expressed as mean±SE. n=6 Swiss albino mice, per group, [b]P<0.05 when compared with *K. parviflora* alone treated and *K. parviflora* treated cyclophosphamide immunosuppressed group (19 days). *K. parviflora*: *Kaempferia parviflora*; SE: Standard error

Table 6: Effect of ethanol extract of *Kaempferia parviflora* on the hemagglutination titer in cyclophosphamide immunosuppressed Swiss albino mice

Days	A_I	A_{II}	A_{III}	A_{IV}
0th day	14.667±1.33	12.000±1.79	12.000±1.79	12.000±1.79
12th day	48.000±7.16	6.667±0.84	938.667±85.33[a]	938.667±85.33[a]
19th day	53.333±6.76	8.000±1.79	298.667±42.67[b]	149.333±21.33[b]

Value are expressed as mean±SE. n=6 Swiss albino mice, per group, [a]P<0.05 when compared with cyclophosphamide control (12 days); [b]P<0.05 when compared with cyclophosphamide control (19 days). SE: Standard error

Table 7: Effect of ethanol extract of *Kaempferia parviflora* on the bone marrow cellularity in cyclophosphamide immunosuppressed Swiss albino mice, millions

Days	A_I	A_{II}	A_{III}	A_{IV}
12th day	4.300±0.17	3.390±0.19	13.900[a]±0.18	4.467[a]±0.16
19th day	3.130±0.14	2.165±0.11	9.250[b]±0.19	7.200[b]±0.51

Value are expressed as mean±SE. n=6 Swiss albino mice, per group, [a]P<0.05 when compared with cyclophosphamide control (12 days), [b]P<0.05 when compared with cyclophosphamide control (19 days). SE: Standard error

Table 8: Effect of ethanol extract of *Kaempferia parviflora* on the total protein and globulin in cyclophosphamide immunosuppressed Swiss albino mice, g/dl

Groups	Biochemical parameters			
	Total protein (g/dl)		Globulin (g/dl)	
	12th	19th	12th	19th
A_I	5.405±0.08	5.425±0.08	2.494±0.04	2.154±0.07
A_{II}	5.606±0.20	4.652±0.11	2.305±0.07	1.873±0.10
A_{III}	6.372±0.27	5.780±0.13[b]	2.973±0.23	2.554±0.16
A_{IV}	5.792±0.07	5.513±0.11[b]	2.857±0.22	2.429±0.14

Total protein: Value are express in mean±SE. n=6 swiss albino mice, per group, [b]P<0.05 when compared with cyclophosphamide control (19 days). Globulin: Value are express in mean±SE. n=6 Swiss albino mice, per group, mean value do not differ significantly. SE: Standard error

Table 9: Effect of ethanol extract of *Kaempferia parviflora* on the foot pad thickness, mm

Days	B_I	B_{II}	B_{II}	B_{IV}
12th day	0.365±0.02	0.337±0.02	1.010[a]±0.05	0.540[a]±0.04
19th day	0.325±0.04	0.280±0.03	0.495[b]±0.05	0.693[b]±0.06

Value are expressed as mean±SE. n=6 swiss albino mice, per group, [a]P<0.05 when compared with cyclophosphamide control (12 days); [b]P<0.05 when compared with cyclophosphamide control (19 days). SE: Standard error

albino mice are the best animal models. Many researchers have used Swiss albino mice to study the immunomodulatory effect.[24,25]

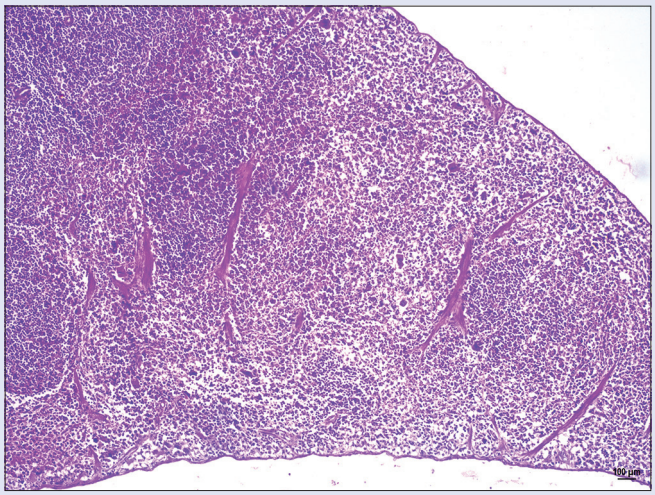

Figure 2: Histopathology of spleen of Swiss albino mice in cyclophosphamide alone-treated group at 12th day. Light microscopic image of H and E-stained sections of spleen. Depletion of lymphocytes in white pulp and marginal zone was observed (×100)

The first six animals in each group were sacrificed on 12th day and the remaining six were sacrificed on 19th day. The study design which included the sacrifice at day 12 and 19 was to assess the humoral immune response by hemagglutination test and cellular immune response by evaluation of bone marrow cellularity. For the evaluation of humoral and cellular immune response, the sacrifice at day 12 and day 19, respectively, is required.

The body weight of the animals and blood parameters like lymphocyte, neutrophils and monocytes may be influenced by external factors such as feed and water. Hence, to know the effect of the drug alone on body weight and blood parameters excluding the influence of external factors, the initial body weight and initial day blood parameters were used as a covariate to eliminate the variations due to influence of initial body weight and blood parameters.[36] Standard error (SE) were calculated separately for each group. We used SE for our data because we are comparing the different groups using mean value and the variation in the mean values is expressed as SE The sample size that we have taken is an estimate of population and hence the estimated variation in mean is expressed as SE. Standard deviation (SD) represents variations in the sample observations while SE represents the variations in the estimate of the population mean. Here, in the study analysis, we have compared the estimate of the population mean, and hence, we have used SE instead of SD.[36]

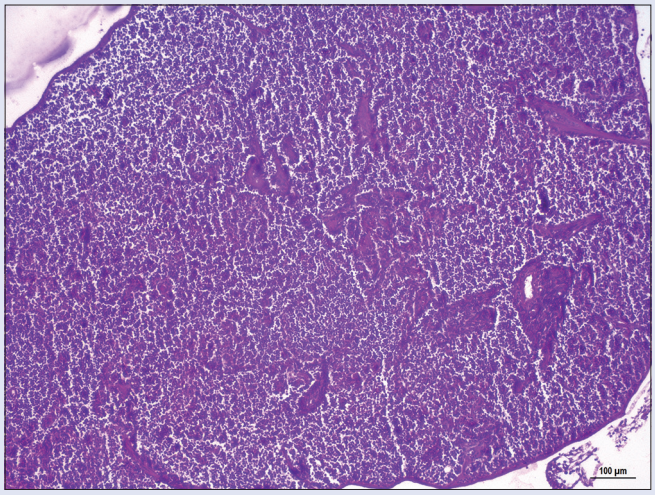

Figure 3: Histopathology of spleen of Swiss albino mice in control group receiving vehicle at 19th day. Light microscopic image of H and E stained sections of spleen. Red pulp and white pulp packed with lymphocytes (×100)

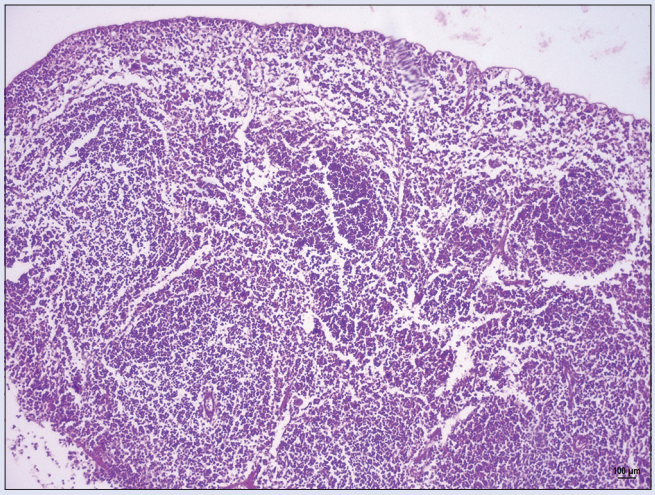

Figure 4: Histopathology of spleen of Swiss albino mice in cyclophosphamide alone-treated group at 19th day. Light microscopic image of H and E-stained sections of spleen. Severe depletion of lymphocytes in white pulp and marginal Zone was noted (×100)

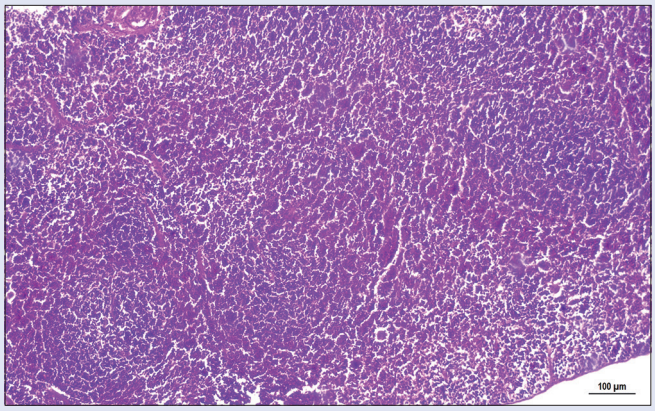

Figure 5: Histopathology of spleen of Swiss albino mice in *Kaempferia parviflora* alone-treated group at 12th day. Light microscopic image of H and E-stained sections of spleen revealed white pulp and red pulp with proliferation of lymphocytes (×100)

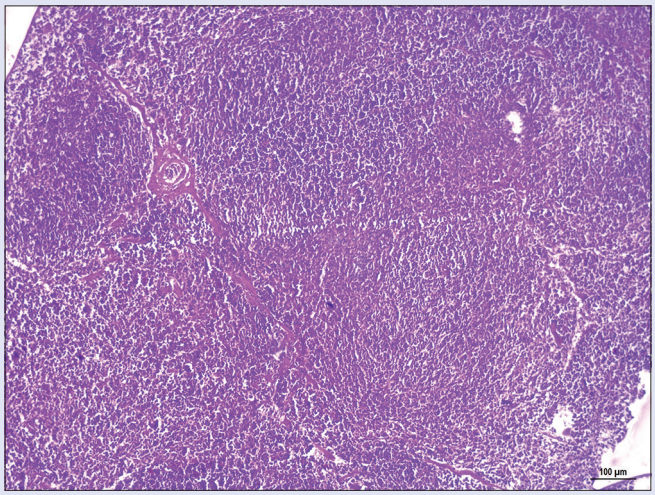

Figure 6: Histopathology of spleen of Swiss albino mice in *Kaempferia parviflora* alone-treated group at 19th day. Light microscopic image of H and E stained sections of spleen. White pulp revealed hyperplasia of lymphocytes with new germinal centre development (×100)

Table 10: Gas chromatography mass spectrophotometry analysis of ethanolic extract of *Kaempferia parviflora*

RT (min)	Name of compound	Relative (%)
19.5	Hexadecanoic acid, ethyl ester	0.72
23.4	Ethyl linolate	0.90
32.0	Techtochrysin	8.73
32.5	Benzaldehyde,(diphenylmethylidene) hydrazone	7.15
32.5	Coumaran-7-ol-3-one, 2-[4-methoxybenzylidene]-6-methoxy-	2.94
35.7	Dimethylchrysin	74.43
37.6	2-(4-Hydroxy-3-methoxyphenyl) -3,7-dimethoxy-4H-chromen-4-one	0.79
40.8	Tri-o-methylapigenin	4.32

RT: Retention Time

Body weight and organ weight

In the present study, there were significant increase in body weight on 12th day and 19th day for all the groups except compared with A_{II}. The increase in body weight was noticed in plant extract-treated cyclophosphamide-induced immunosuppressed mice.[37] The increased in body weight may be due to better feed utilization.[38] Reduction in body weight was observed in cyclophosphamide-treated group in Swiss albino mice.[23]

K. parviflora alone and *K. parviflora*-administered cyclophosphamide-treated immunosuppressed group showed higher relative liver weight when compared with cyclophosphamide control group. Liver is the important organ that responds immediately to any antigen. Relatively higher liver weight when compared with cyclophosphamide control, and similar to that of normal control showed the restoration of normal activity of the liver.[38] Immunocompetency can be viewed as a slight increase in spleen weight,[39-41] and this increase in spleen weight was recorded in *K. parviflora* alone, and *K. parviflora* administered cyclophosphamide-treated immunosuppressed group.[38,42]

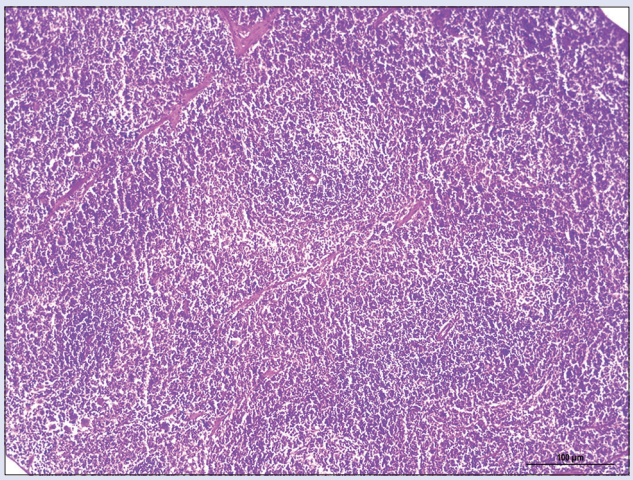

Figure 7: Histopathology of spleen of Swiss albino mice in *Kaempferia parviflora*-treated cyclophosphamide immunosuppressed group at 12th day. Light microscopic image of H and E-stained sections of spleen. The plant extract attenuated the cyclophosphamide-induced lymphocyte depletions in white pulp and red pulp region (×100)

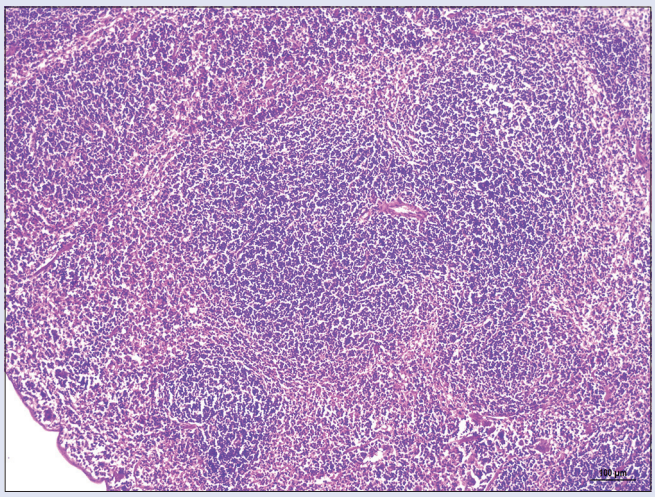

Figure 8: Histopathology of spleen of Swiss albino mice in *Kaempferia parviflora*-treated cyclophosphamide-immunosuppressed group at 19th day. Light microscopic image of H and E stained sections of spleen. Attenuation of cyclophosphamide-induced lymphocyte depletion was noted (×100)

Hematological parameters

In the study, a significant increase in leukocyte count was observed in *K. parviflora* alone and *K. parviflora* administered cyclophosphamide treated immunosuppressed group. Similar works have also shown that there is significant increase in leukocyte count of plant treated cyclophosphamide induced immunosuppressed mice.[43,44] Increased in white blood cell count could be viewed as an important contributing factor in reducing the risk of various diseases.[45]

The major innate of immune cells are phagocytes and lymphocytes and increase in lymphocytes show immunostimulatory effect.[23,46] In the present study, an increased lymphocyte count was observed in *K. parviflora* alone and *K. parviflora* administered cyclophosphamide-treated immunosuppressed mice.[23,46]

Monocyte count was significantly higher in *K. parviflora*-treated immunosuppressed mice when compared with cyclophosphamide control. The innate immune response is strengthened by increase in monocytes.[47] Thus, it can be concluded that *K. parviflora* may have direct stimulating effect on myeloid progenitor cells.

In the present experiment, neutrophil count was found significantly highest in cyclophosphamide control group. The neutrophilia in the cyclophosphamide control group might be a compensatory mechanism to the drop in lymphocyte count observed. Concomitant administration of cyclophosphamide along with plant extract showed a significant decrease in neutrophil count comparable with that of normal group indicating the enhanced efficacy of plant extract in preventing cyclophosphamide induced neutrophilia. Increase in neutrophil count is attributed to marginalization of phagocytic cells, i.e. improved defensive response under normal circumstances as explained by Sultana *et al.*[44] It can be concluded that the plant extract stimulated hematopoietic system by increasing the lymphocytes and decreasing the neutrophil counts.[38]

Immunological parameters

Hemagglutination test

Both A_{III} and A_{IV} showed significant increase in titer value while the lowest value was recorded recorded in cyclophosphamide treated

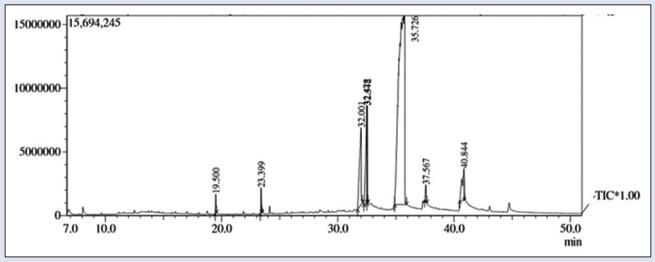

Figure 9: Chromatogram of gas chromatography mass spectrophotometry analysis of ethanolic extract of *Kaempferia parviflora*

group. Increase in proliferation and transformation of B lymphocytes in plasmocytes might increase in antibody titer.[48] The result of the present study suggested the increased immune response when treated with *K. parviflora*.

Bone marrow cellularity

The increase in number of bone marrow cells shows the effect of *K. parviflora* on enhancing immunological response. Bone marrow is a foremost hematopoietic organ. Bone marrow hematopoietic stem cells (HSC) can self-duplicate, proliferate and differentiate to the subordinate cells of positive lineage. Drugs and radiation could damage all the systems, organs and hematopoietic tissues of the whole body. Thus, there was depletion in HSC leading to depletion of mature hematopoietic cells due to administration of cyclophosphamide. The marked increase in bone marrow cell count on administration of *K. parviflora* is an indication of its immunomodulatory property. The administration of chyawanprash and brahma rasayana showed the enhanced level of bone marrow cellularity indicating that hematopoietic cells was stimulated and differentiated.[41] Cyclophosphamide potentially affect the bone marrow cellular production. However, *K. parviflora* extract was found to have a protective effect in cyclophosphamide induced immunosuppression in Swiss albino mice used in the present study.[49]

Biochemical parameters

Serum proteins and globulins are one of the indicators for altered immune status of the individual. So also, serum proteins have role in maintaining homeostasis and resistance to infections.[50] The result on the significantly higher serum protein in *K. parviflora* alone-treated and *K. parviflora*-treated immunosuppressed mice showed the higher immune response of the plant which might have been contributed by the higher values in terms of immunoglobulins and other humoral factors.[51]

Globulins are principally responsible for both the natural and acquired immunity that an individual has against invading organism.[45] The increase in the level of globulin on *K. parviflora* alone-treated mice indicates the antimicrobial action of this plant.

Histopathology of spleen

Depletion of lymphoid cells was observed in white pulp and marginal zone region in cyclophosphamide group. Similar works were observed in experiments conducted by other authors showing immunosuppression.[52] The proliferation of lymphocytes in the white pulp and red pulp in *K. parviflora* alone-treated group and attenuation of cyclophosphamide-induced lymphoid depletion in *K. parviflora*-treated cyclophosphamide immunosuppressed group confirm the immunomodulatory effect of *K. parviflora*.[52,53]

Delayed type hypersensitivity

K. parviflora alone treated and *K. parviflora* treated immunosuppressed mice showed significant increase in foot pad thickness when compared with cyclophosphamide control group. One of the parameter to measure cell mediated immune response is to study the delayed type hypersensitivity in animal model, in which foot pad thickness is measured. Various experiments conducted in animal models had revealed that increase in foot pad thickness indicated cell mediated immunity.[54,55] In the present study, the increased food pad thickness indicated that *K. parviflora* boosted the cell mediated immune response.[56]

Gas chromatography mass spectrophotometry analysis

The major compounds present in the extract were dimethylchrysin, techtochrysin, benzaldehyde, (diphenylmethylidene) hydrazine and tri-o-methylapigenin. The same compounds were previous reported by various authors.[3,17] Tri-o-methylapigenin (synonym: 4',5,7-trimethoxyflavone) was reported to have antiplasmodial, antifungal, and antimycobacterial activity.[3] Techtochrysin, a methoxyflavone, was found to induce skeleton muscle hypertrophy.[57] Flavonoids isolated from different plant sources showed immunomodulatory effect.[58,59] Thus, it could be concluded that the flavonoids such as dimethylchrysin, techtochrysin, tri-O-methylapigenin, 2-(4-hydroxy-3-methoxyphenyl)-3,7-dimethoxy-4 h-chromen-4-one might be responsible for the immunomodulatory activity.

CONCLUSION

The study showed that administration of ethanolic extract of *K. parviflora* boosted the immune response in *in vivo* experiment conducted in Swiss albino mice. Further studies need to be explored to elucidate the exact mechanism of immunomodulatory effect of *K. parviflora*.

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Conflicts of interest

There are no conflicts of interest.

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